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Discovery

Biodegradation potential of *Bacillus lentus* Lp32 isolated from a coal tar contaminated site

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous soil contaminants and they of environmental concern because of their mutagenic effects. In this study, a bacterium capable of growing on Pyrene, a high molecular weight PAH as a sole carbon and energy source was isolated by enrichment technique from a coal tar contaminated soil. The isolate was classified and identified as *Bacillus lentus* strain LP32 on the basis of standard cultural and biochemical techniques. Growth in mineral salts medium supplemented with 100ppm of pyrene resulted in 49.9% degradation in less than 21 days concomitant with exponential increase in cell density and decrease in pH of the culture fluid. The pH of the culture medium dropped from 7.2 to 6.64, the cell population density increased from 2.3 x 10^7 to 4.1 x 10^8 cfu/ml. Similar increases were observed for OD $_{600nm}$ (0.068 to0.573) during the

biodegradation period. These findings may have shown useful catabolic functions of *Bacillus lentus* as an indispensable tool for designing effective bioremediation strategy for decontamination of pyrene polluted sites that are wide spread in our environment.

Keywords: pyrene, biodegradation, bioremediation.

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are pollutants, which are widely distributed in the environment and are detected in air, sediments, surface water, groundwater, and road run off (Khanna et al., 2012). PAHs released into the environment may originate from many sources, including burning of fuel, generation of synthetic fuels from fossil fuels, coal liquefaction and gasification processes, and wood treatment. Many individual PAHs are a serious risk to human health as a result of their carcinogenic and mutagenic potential (Kumar et al., 2006). Since many PAHs have been shown to be carcinogenic, remediation techniques are being developed to remove them from contaminated soil and water. The Physico-chemical parameters of PAHs such as high adsorption coefficient, low water solubility and exhibition of a highly stable complex aromatic structure, reduce the application of usual biodegradation and remediation methods (Jacques, et al., 2007).

At present, many microorganisms are known to be degrading the lower molecular weight PAHs, but little is known on bioremediation of higher molecular weight PAHs (Habe and Omori, 2003). A few hydrocarbon degrading microorganisms degrade some of these non soluble higher molecular weight PAHs by producing surface-active compounds as reported by Das and Mukherjee (2007). However, the enzymatic, metabolic pathways of these lower molecular weight PAHs, like naphthalene, anthracene and phenanthrene have been well acknowledged (Boonchan et al., 2000; Kanaly and Harayama 2000; Habe and Omori, 2003). Moreover, the low rate of microbial degradation of the higher weight PAHs, such as chrysene, benzo[a]pyrene or pyrene has been attributed to its water insolubility (Boonchan et al., 2000; Johnsen et al., 2005).

Pyrene is one of the high-molecular-weight PAHs, which is commonly found in soils contaminated with crude oil, coal tar and other complex mixtures of PAHs (Mohamed et al., 2006; Das and Mukherjee 2007). Its chemical structure is among the several carcinogenic PAHs included in the list of 129 'Priority Pollutants' as compiled by the U.S. Environmental Protection Agency (Habe and Omori, 2003). Biodegradation of Pyrene at the metabolic, genomic and proteomic level by actinomycetes group of bacteria has been reported by (Kim et al., 2007; Khanna et al., 2012). However, information about the occurrence of pyrene biodegraders in some heavily coal tar contaminated sites at PWD works yard along Agege motor road Lagos and Lekki both in Lagos state Nigeria is a meager. According to Habe and Omori (2003) there may still be many unidentified PAH degrading bacteria. The aim of the present study was to isolate bacterial isolates from these heavily coal tar-contaminated soils with ability to grow in aqueous medium and utilize pyrene. It is also important to isolate and characterize bacteria in contaminated soils capable of mineralizing high molecular weight PAHs since the isolates could be used at bioremediation sites to improve the overall PAH degradation rate.

2. MATERIALS AND METHODS

The study areas were road construction sites at PWD works yard along Agege motor road Lagos and Lekki both in Lagos state Nigeria. The sampling points were chosen because they have been heavily polluted with coal tar for some years before this research work. The PWD works yard is between longitude 3.35479E and latitude 6°.527378N and has a length of 2.62 kilometers, while the Lekki site lies between latitude 6° 23′ N and longitude 4° 13′E of Lagos State Nigeria. Sampling locations were identified with a hand-held global positioning system (GPS 12 Garmin model).

Polluted soil samples for microbial isolations and physico-chemical parameters were collected (up to the depth of 10cm) randomly from PWD works yard and Lekki sites. The samples were placed into sterile bottles and transported immediately to the laboratory for further work. The physico-chemical composition of the soil was determined using standard protocol of (Bray and Kurtz, 1945).

Total heterotrophic bacteria were obtained by aseptically introducing aliquots (0.1 mL) of appropriate dilutions on nutrient agar plates incubated at 30°C for 24hours. The population of hydrocarbon-utilizing bacteria was determined by plating 0.1 mL aliquots of appropriate dilution on minimal salt agar (MSA). MSA was composed of MgSO4.7H2O, 0.2; Na_2HPO_4, 2.13; NH_4CL, 0.5;KH_2PO_4, 1.3; yeast extract, 0.055; trace elements, 1; agar, 15; pH 7.0 + 0.2 (Kastner *et al*., 1994). The medium was sterilized by autoclaving at 121°C for 20 min in all tests *in vitro*. Crude oil was supplied by vapour phase transfer as described by (Amund and Adebiyi, 1991). Incubation was done at 30°C for 5-7 days.

The hydrocarbon-utilizing bacterial species were tested for their ability to grow on a variety of carbon sources. Test compounds (energy source at 0.1% (w/v or v/v) were added to minimal salts medium (MSM) was composed of (g L-1 of deionized water): MgSO4.7H2O, 0.2; Na_2HPO_4, 2.13; NH_4CL, 0.5;KH_2PO_4, 1.3; yeast extract, 0.055; trace elements, 1; agar, 15; pH 7.0 + 0.2 previously by Kastner *et al*., (1994) as a sole carbon. Volatile compounds were supplied to the microorganisms in the vapor phase. Microorganisms were inoculated and incubated with constant sharking at 30°C for 14 days. The evaluation of growth tests was carried out by visual monitoring and scored relatively. Growth test were conducted in triplicate of each substrate.

Aerobic cultures were grown in MSM. The medium was sterilized by autoclaving at 121°C for 20 min in all tests *in vitro*. Soil (1 g) was added to 99 mLs sterilized Ms medium in a dark flask and supplemented with (100 ppm) pyrene as the sole carbon and energy source. Enrichment flasks were incubated at 30°C (room temperature) on a rotary shaker at 150rpm in darkness for 14 days. Utilization of the compound in enrichment cultures was evidenced by a visual increase in turbidity. When growth had occurred, the enrichment culture was transferred to fresh sterilized MSM using 1% inoculum and incubation continued. This procedure was repeated for three successive transfers. Pure cultures were isolated from enrichments by plating out appropriate dilutions onto nutrient agar plates. Single colonies were transferred to MSM containing pyrene to confirm their ability to grow on this substrate. Isolates able to utilize pyrene (as described below) were selected for further characterization and study. Bacterial isolates were preserved in Luria Bertani (LB) agar slants and kept at 4°C prior to use.

Chemicals Pyrene (95% purity) was purchased from sigma chemical Co. (Steinheim, Germany). All other chemicals and solvents used were high purity grade reagents.

Bacteria were identified based on phenotypic identification using previously established schemes (Holt *et al*., 1994). They were further confirmed using the API 20E (Biomerieux SA, France) kit according to the manufacturer's instruction.

The bacterial species were tested for their ability to utilize pyrene as a sole carbon source. Test compound was added to MSM as a sole carbon and energy source at 100 ppm. Microorganisms were inoculated with constant shaking at 30 °C for 14 days. The evaluation of growth tests was carried out by visual monitoring and scored relatively. Growth test were conducted in triplicate of each substrate.

Growth at the expense of pyrene was verified by demonstrating an increase in bacterial cell concentration concomitant with a decrease in pyrene concentration. Overnight culture of strain LP32 grown on LB plates for 24 hours were suspended in minimal salt medium (A_{600} 0.5*)*, and the suspension was used as an inoculums (0.5 ml). Replicate cultures were grown in 250 ml conical flasks containing 20 ml of minimal medium and (100 ppm). Incubation was performed at 30 °C in the dark for 21 days with constant shaking. Flasks inoculated with dead cells of strain LP 32 served as control. Biodegradation was monitored at three-day intervals. The utilization of pyrene by the microbial isolate was evaluated by monitoring bacterial growth measured by total viable counts on nutrient agar, changes in pH, the optical density at 600 nm with a Milton Roy spectronic 20D spectrophotometer, and Gas chromatograph.

Pyrene was extracted from bacterial culture fluids with methlene chloride. Gas chromatographic analysis of methlene chloride and of pyrene was performed on a Hewlett Packard model 5890 gas chromatogram equipped with a flame ionization detector, using a 30 m x 0.25 mm [inside diameter]; 0.25 µm film thickness. Hewlett Packard-5 fused silica capillary column programmed at 60°C for 2 min, holding at 15°C/ min to 4 min and for 8 min. Injector detector temperature were set at 300 and 320°C, respectively. Flame ionization was used to detect and quantitate the compounds eluting from the chromatographic column. The carrier gas was nitrogen. The data were digitized using a model 760 Nelson Analytical A/D converter. Data acquisition, chromatogram processing, and correlation were completed on Hewlett Packard 9000/300 computer using chevron software data weight acquired with Hewlett Packard series 11 running on chem. station software.

The data obtained were analysed using the prism version 5.03 statistical software programmes (Graph pad software, San Diego, CA. USA). Descriptive statistics including percentages were used to summarize the data.

3. RESULTS

The physico-chemical characteristic of the PWD works yard and Lekki coal tar polluted soil samples are shown in (Table 1). The physical appearance of the soil samples revealed dark, sticky and oily for both PWD works yard and Lekki. More so, the pH of the soil samples tested showed an average of 6.3 and 6.4, respectively. Furthermore, the percentage of organic matter content in the soil samples was 0.30 and 0.28% respectively. However, percentage moisture, total phosphorus percentage and total nitrogen percentage were 8.19, 0.091 and 0.022 %, respectively for PWD works yard soil samples while Lekki samples revealed 8.15, 0.081 and 0.014 %, respectively.

Table 1 Physico-Chemical Composition of PWD works yard and Lekki coal tar polluted soil samples

PARAMETER	MEAN DETERMINATION	
	PWD coal tar polluted soils	LEKKI coal tar polluted soils
Physical appearance	Dark, sticky and oily	Dark, sticky and oily
pH	6.3	6.4
Organic matter (%)	0.30	0.28
Moisture (%)	8.19	8.15
Total phosphorus as $P_2 O_5$ (%)	0.091	0.081
Total Nitrogen (%)	0.022	0.014

Table 2 Bacterial population densities of coal tar polluted soil samples

Location	Bacteria (cfu/ mL)	Hydrocarbon-utilizer (cfu/ mL)	Hydrocarbon-utilizing percentage (%)
PWD works yard	2.8×10^9	5.4×10^6	0.59
Lekki	2.8×10^9	6.3×10^6	0.67

The results of the total heterotrophic and hydrocarbon-utilizers in the polluted soil are represented in Table 2. The result show that soil samples from PWD had an average of 2.8×10^9 cfu/ mL with 5.4×10^6 cfu/mL hydrocarbon utilizers. The Lekki site had an average of 2.8×10^9 cfu/ mL with 6.3×10^6 cfu/mL hydrocarbon utilizers. The percentage of the hydrocarbon utilizer for PWD was 0.59% while Lekki site revealed an average of 0.67%.

After three successive transfers in MSM containing crude oil, four organisms were isolated. The organisms were identified as B2 *Pseudomonas stutzeri;* A81 *Acinetobacter calcoaceticus;* LP32 *Bacillus lentus* and LP31 *Pseudomonas aeruginosa.*

The isolates were able to grow on a broad range of substrates supplied as sole carbon and energy sources (Table 3). Compounds utilized included toluene, crude oil, diesel oil, engine oil with exception of *Pseudomonas stutzeri* which grew on engine oil. Kerosene was readily utilized by *Acinetobacter calcoaceticus* and *Pseudomonas stutzeri* while, *Pseudomonas aeruginosa* and *Bacillus lentus* grew slightly on this substrate. The PAHs used were anthrancene, pyrene and phenanthrene. Only *Pseudomonas aeruginosa* utilized anthrancene moderately well while growth of other strains were poor. *Bacillus lentus* strain utilized pyrene and phenanthrene extensively more than other isolates.

After three successive transfers in MSM containing pyrene, isolation procedures produced two organisms. The two isolates LP31was identified as yellow pigmented *Pseudomonas aeroginosa* while LP32 was identified as *Bacillus lentus*.

Table 3 Growth on different carbon sources

	Toluene	Crude oil	Diesel oil	Engine oil	Kerosene	Anthracene	pyrene	phenanthrene
A81	+++	+++	+++	+++	+++	+	+	+
B2	+++	+++	+++	+	+++	+	+	+
LP32	+++	++	+++	++	+	+	+++	+++
LP31	+++	+++	++	+++	+	++	+	+

Key- no growth, + poor growth, ++ moderate growth, +++ heavy growth

Table 4 Pyrene utilization test

ORGANISM	PYRENE UTILIZATION
Pseudomonas aeroginosa	+
Bacillus lentus	+++

KEY: no growth, +, poor growth, +++, heavy growth

Table 5 Growth Kinetics of Pyrene by *Bacillus lentus*

Isolate	Substrate	specific growth rate (h^{-1})	Mean generation time (Day)	Percentage degradation
Bacillus lentus	pyrene	0.156	4.46	49.9

These organism were found to be pyrene degraders on further testing, it was found that *Bacillus lentus* had higher potential to utilize pyrene as carbon source. Based on this, strain *Bacillus lentus* was selected for further studies. The abilities of these organisms to utilize pyrene were indicated by increases in turbidity (Table 4).

Figure 1 Gas chromatographic profile for pyrene on the day 0 inoculated with dead calls of *Bacillus lentus* strain LP32

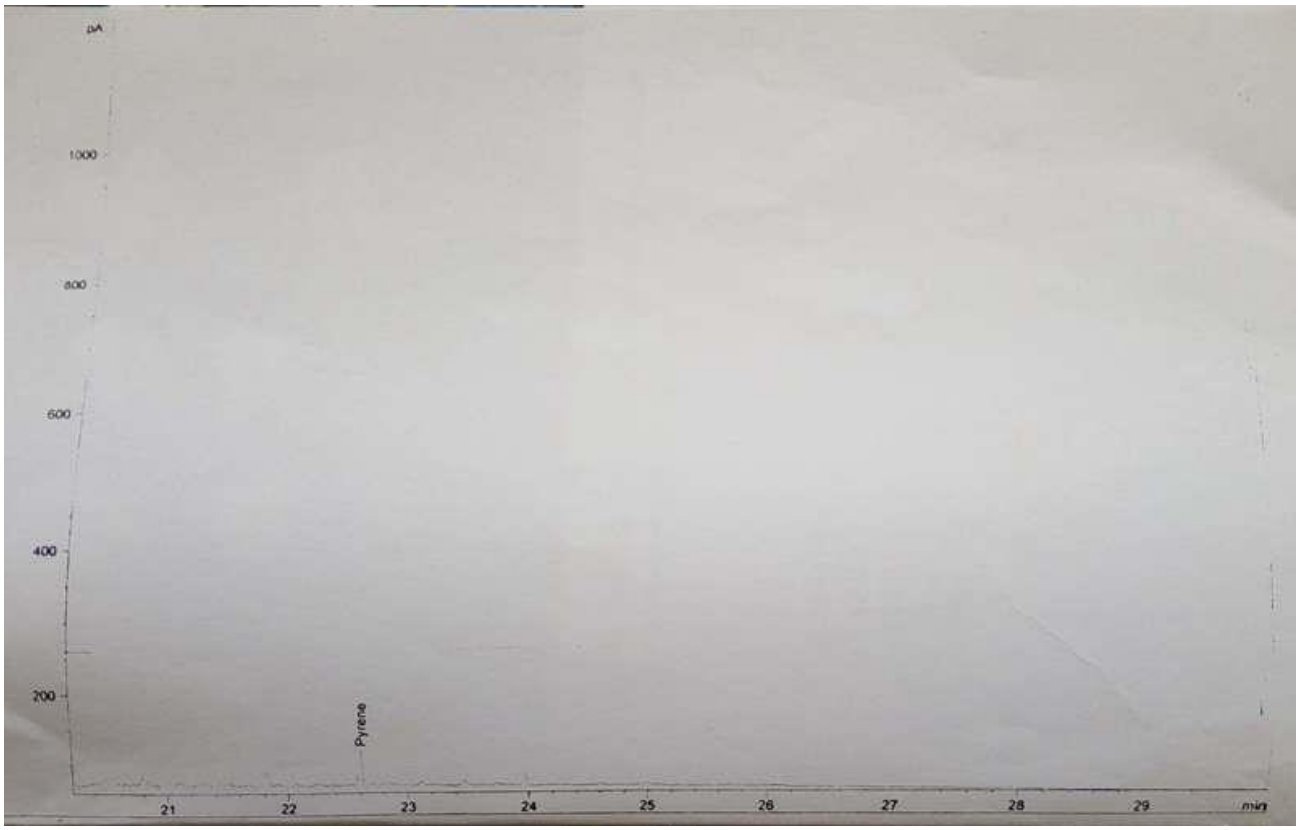

Figure 2 Gas chromatographic profile for pyrene recovered during 21 days aerated batch culture of *Bacillus lentus* strain LP32

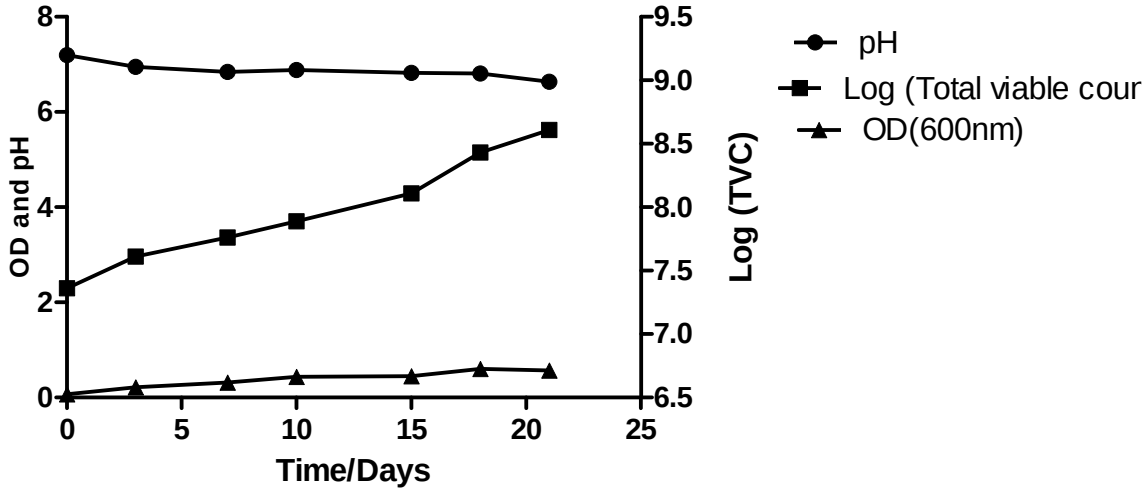

Figure 3 Growth profile of *Bacillus lentus* in minimal salts medium supplemented with pyrene as sole carbon source and energy

The role of *Bacillus lentus* in the degradation of pyrene was investigated and the results are presented in Table 5 and Figs. 1-3. *Bacillus lentus* degraded 49.9% of 100ppm pyrene within 21 days as indicated by GC analysis. Pyrene degradation was accompanied by a rapid increase in total viable counts with no lag phase from 2.3 x 10^7 to 4.1 x 10^8 cfu/ml in 21 days. There was also a corresponding increase in the optical density (OD) reading of the culture from 0.068 to 0.572 whereas, the growth of the organism resulted in a gradual decrease in pH of the culture medium from 7.20 to 6.64. Over the cause of study, the pyrene crystals were

degraded and eventually disappeared with prolongs incubation. There was a change in color of the culture medium after about 5 days of incubation from colorless to yellow.

4. DISCUSSION

To have a successful bioremediation technique on a polluted soil, proper understanding of the properties of the contaminated site is required. The physico-chemical composition of the soil used as the inoculums, revealed relatively low concentrations of nitrogen (N) and phosphorus (P), elements required for efficient and normal functioning of microbial cells (Andrew and Jackson, 1996). In the absence of sufficient nitrogen and phosphorus pollutant degradation reactions may be slow even though carbon and energy sources required for growth are available (Vidali, 2001). This may partially explain the persistence of PAHs in the soil in spite of the presence of a capable microbial population.

Four hydrocarbon utilizing bacterial strains were isolated and characterized based on morphological characteristic and biochemical tests. They were subsequently identified as *Acinetobacter calcoaceticus* A81, *Pseudomonas stutzeri* B2, *Pseudomonas aeruginosa* LP31 and *Bacillus lentus* LP32. All the isolates were aerobic. Many aerobic bacteria have the ability to utilize hydrocarbon mostly n-alkanes homoloques (Das and Mukherjee, 2007).

In this study, two pyrene utilizing bacterial strains were successfully isolated and characterized based on morphological characteristic and biochemical tests. They were subsequently identified as *Bacillus lentus* strain LP32 and *pseudomonas aeroginosa* strain LP31. All the isolates were aerobic. PAH-contaminated soils often provide a broad range of substrates and metabolites that form an environment for a complex microbial community. Bacterial communities in contaminated soils tend to be dominated by the strains that can survive in the polluted soil and thus are able to utilize the contaminant as carbon and energy source (Zucchi *et al.* 2003).

Pyrene utilization by microorganisms could be due to the synergistic effect of various microorganisms that may have a wide range of substrate specificity (Boonchan *et al.*, 2000). The bacterial strains isolated from this present work were able to grow on pyrene supplied as sole carbon and energy source. *Bacillus lentus* strain LP32 utilized pyrene more extensively than the other isolates. Based on this, strain LP32 was selected for further studies on pyrene. Members of the genus *Bacillus* have been used and reported in past research works for PAH biodegradation. Das and Mukherjee, (2007) reported differential utilization of pyrene as the sole source of carbon by *Bacillus subtilis* and *Pseudomonas aeruginosa.* Jacques, *et al.*, 2007; Lin and Cai, 2008 and Toledo *et al.* (2006) have also majorly attributed *Bacillus* strains with the property to colonize environments contaminated with PAHs. Mohamed *et al.* (2006) also isolated *B. firmus* from PAH contaminated soils in Kuwait as a bacterial degrader.

The microbial degradation of pyrene has been observed by a number of investigators (Wang and Bartha 1990; Weissenfels *et al.*, 1991; Nwinyi *et al.*, 2013), but only a few reports describe the degradation of pyrene as a sole carbon source (Heitkamp *et al.*, 1988; Walter *et al.*, 1991; Kastner *et al.*, 1994; Nwinyi *et al.*, 2013). The test organism strain LP32 on mineral salts medium supplemented with a concentration of 100 ppm pyrene in less than 21 days as indicated by GC analysis revealed 49.9% of degradation with a growth rate of 0.156 h^{-1} even faster than that of other microorganisms. Tulbanit *et al.*, (1996), reported growth rate of 0.014 and 0.013 h^{-1} for *Pseudomonas sp*. K-12 and B-24 respectively. Boldrin *et al.*, (1993) reported a growth rate of 0.056 h^{-1} for a *Mycobacterium sp.* Grown on pyrene, and Walter *et al.*, (1991) revealed that *Rhodococcus* strain UWI had a growth rate 0.023 h^{-1}. *Mycobacterium sp.* BB1, *Mycobacterium sp.* VFI and *Gordona sp*. BP 9 were isolated from soils contaminated with fuel or coal tar oil and found to be capable of growing on a solidified mineral medium sprayed with pyrene as the sole carbon source (Kastner *et al.*, 1994).

5. CONCLUSION

In the present study, *Bacillus lentus* strain LP32 which is capable of degrading pyrene was isolated and identified. However, it was observed that the potentials of strain LP32 may be exceptional in designing effective bioremediation strategy for decontamination of PAH –contaminated sites. In soils such as those from which the isolates were obtained, it may be necessary to adjust nitrogen and phosphorus concentrations to enhance biodegradation of the organic pollutant.

SUMMARY OF RESEARCH

1.To have a successful bioremediation technique on a polluted soil, proper understanding of the properties of the contaminated site is required.

2.In this study, two pyrene utilizing bacterial strains were successfully isolated and characterized based on morphological characteristic and biochemical tests. Bacterial communities in contaminated soils tend to be dominated by the strains that can survive in the polluted soil and thus are able to utilize the contaminant as carbon and energy source.

3.Pyrene utilization by microorganisms could be due to the synergistic effect of various microorganisms that may have a wide range of substrate specificity.

FUTURE ISSUES

Biodegradation is the major indulgence for most organic contaminated soil environments. On the other hand, the microbial activities are dependent upon availability of relevant nutrients for degradation. PAH-polluted soils usually provide a broad range of substrates and metabolites there by creating an environment that forms a complex microbial community. More so, the good strains of bio-degraders in contaminated soils tend dominated the other microbial strains because they can survive the environmental toxicity at that time and thus utilize the contaminant itself for growth.

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